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MOLECULAR GENETICS OF AUXIN SIGNALING

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■ **Abstract** The plant hormone auxin is a simple molecule similar to tryptophan, yet it elicits a diverse array of responses and is involved in the regulation of growth and development throughout the plant life cycle. The ability of auxin to bring about such diverse responses appears to result partly from the existence of several independent mechanisms for auxin perception. Furthermore, one prominent mechanism for auxin signal transduction involves the targeted degradation of members of a large family of transcriptional regulators that appear to participate in complex and competing dimerization networks to modulate the expression of a wide range of genes. These models for auxin signaling now offer a framework in which to test how each specific response to auxin is brought about.

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INTRODUCTION

Reviews about auxin traditionally start with a sentence about how very important auxin is to plant growth and development, followed by a despairing comment about how, despite more than a century of research, we know very little about how it works (28, 38). Certainly there has been plenty of time to establish a very complex phenomenology for auxin biology. Exogenous addition of auxin to plants, plant tissues, and plant cells elicits a multitude of responses (7, 16). These include

changes in the transcription of various gene families, a range of electrophysiological responses, changes in the rates of cell division and cell elongation, and changes in tissue patterning and differentiation. Which response is triggered depends on a wide variety of factors including cell type, developmental stage, environmental conditions, and the concentration of auxin added. In untreated plants, a similarly wide range of events can be correlated with changes in auxin levels, sensitivity, or transport. This striking range of responses has been central to the auxin mystery. Is it that auxin does just one thing that is linked in an unknown way to all these different responses? Or is it that this simple molecule has astonishing biochemical multifunctionality? Recent progress in understanding the molecular mechanism of auxin signaling is beginning to answer these questions, and the answer seems to be that both explanations are to some extent correct.

AUXIN PERCEPTION

Auxin signaling is assumed to start with the perception of auxin by its interaction with some kind of receptor. Evidence suggests that there are multiple sites for auxin perception, and in this sense, auxin can be considered to be multifunctional in that the auxin signal appears to be transduced through various signaling pathways.

Auxin Binding Protein 1

The search for auxin receptors has naturally focused on the isolation and characterization of proteins that bind auxin (reviewed in 101). Although a variety of such proteins has been identified, conclusive evidence linking them to auxin responses has proved difficult to obtain, and the biochemistry of the proteins has been, depending on your perspective, intriguing, perplexing, or frustrating.

The best-characterized auxin binding protein is ABP1 (reviewed in 73), which was first described in maize (35). Excitement about the role of ABP1 in auxin perception is driven by the high specificity and affinity of its auxin binding, with a K_D for the synthetic auxin NAA of 5×10^{-8} M (73). However, almost none of its other properties are characteristic of a typical receptor. The protein has no homology to any other known receptor family, and the vast majority of it is retained in the endoplasmic reticulum, where the pH is too high for strong auxin binding (34, 92). Some ABP1 apparently escapes to the plasma membrane, where it mediates several cellular responses to applied auxin, including tobacco mesophyll protoplast hyperpolarization (57, 58), the expansion of tobacco and maize cells in culture (10, 44), tobacco mesophyll protoplast division (24), and stomatal closure (26). It is clear that ABP1 acts at the cell surface to mediate these responses because the exogenous addition of anti-ABP1 antibodies, which are unable to enter the cell, can interfere with the ability of auxin to induce the responses. In whole plants, transgenic approaches to change ABP1 levels have resulted in relatively modest phenotypic effects (5, 44). Phenotypes are in general limited to effects on the balance between cell division and cell expansion. For example, overexpression of

ABP1 in tobacco plants results in increases in leaf mesophyll cell size, without affecting final leaf size (44).

Many aspects of ABP1 biology remain mysterious, but recently two extremely important tools have been added to the collection available for the investigation of ABP1 function. First an insertional mutant in the *Arabidopsis ABP1* gene has been recovered, allowing the phenotype of complete loss of ABP1 function to be assessed for the first time (10). The phenotype of plants homozygous for the mutation is embryo lethality early in the globular stage. This demonstrates the essential role that ABP1 plays in plant growth, but it makes analysis of the postembryonic role of ABP1 difficult, requiring conditional mutations.

The second major advance is the solving of the crystalline structure of ABP1 to a resolution of 1.9 Angstroms (107). The combination of these new genetic and biochemical tools will allow better analysis of the events immediately up- and downstream of ABP1 (103) so that its role in auxin signaling can be clarified.

Intracellular Sites for Auxin Perception

The existing evidence suggests that there are multiple auxin receptors, and hence the work on ABP1 is expected to answer only part of the question of how the auxin signal is perceived. For example, although ABP1 appears to act at the cell surface, there is good evidence for intracellular perception of auxin, much of which is derived from comparing the effects of auxins that differ in their transport properties into and out of cells (e.g., 12). This approach has been strengthened by the isolation of mutants in *Arabidopsis* that differ in their ability to transport auxins. For example, the *AUX1* gene of *Arabidopsis* encodes a protein with homology to amino acid permeases and is thought to act as an auxin uptake carrier (6, 65). Loss of *AUX1* function results in a variety of phenotypes including auxin-resistant root elongation and reduced root gravitropism (6, 65, 81). The roots of *aux1* mutants are resistant to the effects of membrane-impermeable auxins such as 2,4-D. However, *aux1* mutants respond normally to the membrane-permeable auxin NAA, and addition of NAA to *aux1* mutant roots can restore graviresponse (65, 111). This suggests that intracellular auxin is important for root growth inhibition.

Potential intracellular auxin binding proteins have been identified. For example, a 57-kDa soluble auxin binding protein has been identified in rice (47). This protein appears to interact directly with the plasma membrane proton pumping ATPase, suggesting a very short signal transduction pathway from auxin to increased proton pumping, cell wall acidification, and hence cell elongation (48, 49).

Other Routes for Auxin Perception

There is considerable speculation about the possible role of auxin transporters in auxin signaling (for a review of auxin transport see 78). These proteins most certainly interact with auxin, and it is possible that auxin levels in the cell are monitored by measuring the flux of auxin through either influx or efflux carriers, or both, like counting sheep through a gate. It is also possible that specific transporter

family members do not in fact act as transporters but rather have specialized receptor function. An interesting precedent is seen in the budding yeast *Saccharomyces cerevisiae*, where recent evidence suggests that amino acid levels are detected by a permease-like receptor, Ssy1 (22). That auxin signaling evolved from an amino acid signaling pathway is an attractive hypothesis, and hence it is interesting to note that the budding yeast amino acid signal is transduced from Ssy1, via a pathway dependent on regulated protein stability, to bring about changes in transcription (8, 40). Strong evidence to support a role for a very similar signal transduction pathway acting in the auxin response is now available (see below).

AUXIN SIGNAL TRANSDUCTION

Although there is currently little to link auxin perception with downstream events, rapid progress in the area of auxin signal transduction has been made recently through the use of genetic approaches in *Arabidopsis* (reviewed in 59). Large screens for mutants with altered auxin sensitivity were used to define genes whose normal function is required for wild-type auxin response. Among the loci defined by these screens are *AXR1*, *AXR2*, *AXR3*, *AXR4*, and *AXR6*. A sixth locus, *TIR1*, was originally identified because mutations in it result in resistance to inhibitors of polar auxin transport, but these mutations were subsequently found also to confer auxin resistance (85). The molecular basis for the *axr4* and *axr6* phenotypes (36, 37) has not yet been determined, but the remaining loci have been cloned and they appear to function in a single pathway involved in auxin-regulated ubiquitin-dependent protein turnover (Figure 1).

The Role of Ubiquitin-Mediated Protein Degradation

Ubiquitin-mediated proteolysis occurs through the conjugation of a multiubiquitin chain to target proteins, which are subsequently degraded by the 26S proteasome (reviewed in 43, 80, 102). Conjugation of ubiquitin is a three-step process. Ubiquitin first is activated by ubiqutin activating enzyme, E1. This requires ATP and results in the formation of a high-energy thiol ester linkage between ubiquitin and a conserved cysteine in the E1. Ubiquitin is then passed to a ubiquitin conjugating enzyme, E2, which acts in concert with a ubiquitin protein ligase, E3, to link ubiquitin to a lysine residue of the target protein. A fourth step in which a multiubiquitin chain is extended from this first ubiquitin may require a multiubiquitin chain assembly factor, E4 (4, 54).

Much of the substrate specificity in the selection of proteins for degradation lies in the E3 enzyme, and this is reflected in the fact that hundreds of E3s of various classes are encoded by the eukarytotic genomes so far sequenced (21, 80, 102, 110). Prominent among these are the so-called SCF-type E3s (reviewed in 21, 110). SCFs take their name from three of their subunits; they are multimeric enzymes consisting of at least four subunits. Two of these subunits, members of the Cullin (also known as CDC53) and RBX1 (also known as ROC1 or HRT1) families,

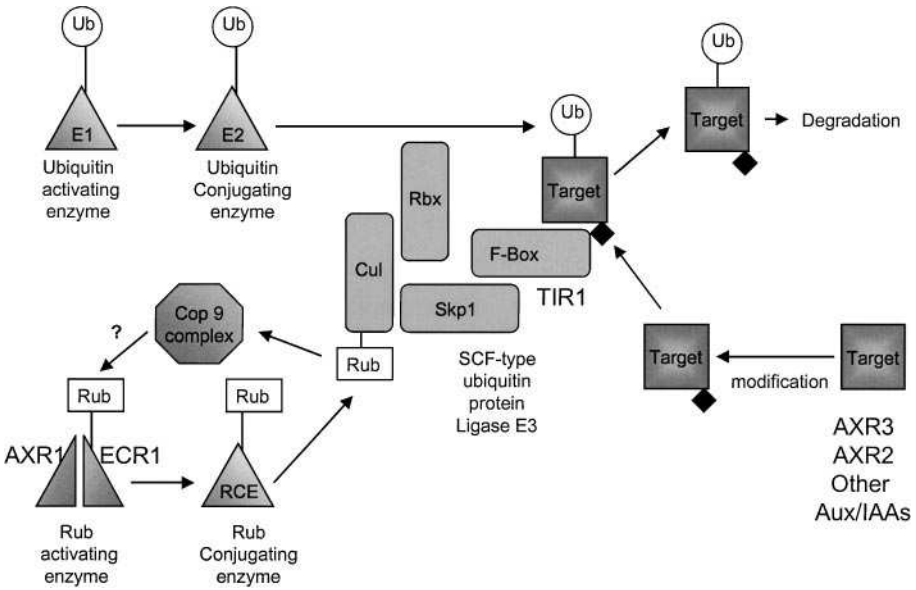

Figure 1 Auxin regulates the ubiquitination of target proteins, marking them for degradation by the 26S proteasome. The figure shows key components of this pathway. Ubiquitin (Ub) must be activated before conjugation to specific targets (*top left*). Target selection is mediated by the F-box–containing subunit of an SCF-type ubiquitin protein ligase (*center*). Auxin-regulated modification of the targets, which include the Aux/IAA proteins, is likely to be required for recognition by the F-box protein (*right-hand side*). Efficient activity of the SCF requires conjugation and deconjugation of a ubiquitin-related protein of the Rub family to the Cullin (Cul) subunit of the SCF (*bottom left*). Like ubiquitin, Rub must be activated before conjugation by a dimeric enzyme with homology to ubiquitin-activating enzyme. Deconjugation of Rub requires the Cop 9 complex. It is not clear if Rub protein is recycled during this process. In *Arabidopsis*, mutations, including *axr1*, *ecr1*, *axr2*, *axr3*, and *tir1*, in components of this pathway result in defective auxin response.

form a dimer able to catalyse multiubiquitin chain formation (88). Consistent with this function, RBX1 family members contain a RING-H2 finger domain common to many ubiquitin ligases (21). The Cullin/RBX1 dimer is linked to an F-box–containing protein through a member of the SKP1 protein family (15, 50, 79). The amino-terminal F-box motif, characteristic of F-box proteins, is required for the interaction between the F-box protein and the SKP1. The carboxyl-terminus of the F-box protein consists of any one of a variety of protein-protein interaction domains, and this domain interacts with the ubiquitination target and hence selects the substrate for degradation (50, 79). SCF-dependent signaling pathways are found throughout the plant, fungal, and animal kingdoms (15, 19, 21, 43, 79). For example, as mentioned above, in budding yeast, amino acid signaling is

mediated through targeted degradation of transcription factors by an SCF complex including the F-box protein Grr1 (8, 40).

Crucial evidence for the involvement of this type of regulated protein turnover pathway in auxin signaling came when the *TIR1* gene was cloned and found to encode an F-box protein (85). TIR1 has subsequently been shown to participate in an SCF complex, SCF^{TIR1}, supporting the hypothesis that targeted protein degradation is required for normal auxin signaling (27). The auxin resistance phenotype conferred by complete loss of TIR1 function is relatively weak, but sequence comparisons with other *Arabidopsis* F-box proteins have identified several close homologues of TIR1, suggesting that TIR1 is likely to be functionally redundant in part (85, 110). The carboxyl-terminal domain of TIR1 and its close homologues consist of leucine-rich repeats, which are presumably involved in target selection (39, 50). So what then are the targets for SCF^{TIR1}? Recent evidence suggests that the products of the *AXR2* and *AXR3* genes interact with SCF^{TIR1} and are destabilized as a result.

SCF^{TIR1}-Mediated Instability of the Aux/IAA Protein Family

The *AXR2* and *AXR3* loci were defined by dominant mutations that confer a range of auxin-related phenotypes (61, 95, 96, 106). Both genes have been cloned and found to encode members of the Aux/IAA gene family (72, 84). Aux/IAA proteins are found throughout higher plants and are characterized by four highly conserved domains (reviewed in 3, 31) (Figure 2). The dominant mutations in the *axr2* and *axr3* alleles map to domain II (72, 84). Similar dominant mutations in domain II of other Aux/IAA family members have been recovered from a range of screens for auxin-related phenotypes such as tropism defects, photomorphogenesis defects, and altered root branching patterns (83, 93). The generality of these results indicates that domain II is of great importance for wild-type Aux/IAA function.

Several lines of evidence demonstrate that domain II functions in protein destabilization. Those Aux/IAA proteins that have been examined in detail are localized in the nucleus and have very short half-lives, ranging from a few minutes to a few hours (2, 29, 77). The fusion of Aux/IAA proteins to entirely unrelated reporter proteins, such as luciferase or beta glucuronase (GUS), results in the destabilization of the reporter protein (29, 108). This indicates that the Aux/IAAs contain a transferable destabilization sequence, a so-called degron. This degron has now been further defined as a 13–amino acid region from the core of domain II because fusion of just these 13 amino acids to luciferase is sufficient to confer a short half-life on the luciferase protein, providing that it is localized to the nucleus (82). A second line of evidence for the importance of domain II in regulating Aux/IAA stability comes from the analysis of the molecular basis for the phenotypes conferred by *axr3-1*, *axr2-1*, and similar Aux/IAA mutants. The dominant domain II mutations found in such alleles increase protein half-life dramatically, in a range of direct and indirect assays, without affecting nuclear localization (29, 77, 108). These data confirm that domain II and nuclear localization are necessary and sufficient to give the Aux/IAA proteins their short half-life and that this rapid degradation is required for a wild-type auxin response.

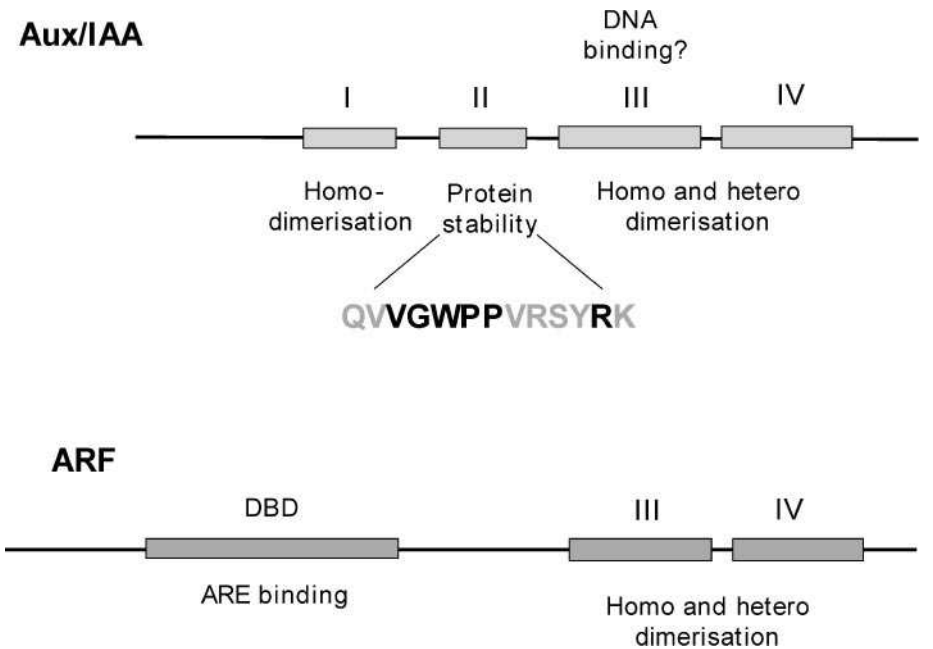

Figure 2 The linear structure of typical family members of the Aux/IAAs (*top*) and auxin response factors (ARFs) (*bottom*). The proteins share homology at their carboxyl termini in two domains (III and IV), which mediate homo- and heterodimerization. At the amino terminus, Aux/IAA proteins have two domains, one of which (II) is necessary and sufficient to mediate auxin-regulated destabilization of the protein. The consensus sequence of this domain is shown, with the invariant amino acids in black. ARFs have an amino-terminal DNA binding domain that binds to the auxin response elements (AREs) of auxin-regulated genes.

The requirement for both functional SCF^{TIR1} and rapid turnover of Aux/IAA proteins for normal auxin signaling has led to the hypothesis that Aux/IAAs are targeted for degradation by SCF^{TIR1}. Consistent with this idea, inhibitors of the 26S proteasome stabilize Aux/IAA-reporter fusion proteins, indicating that Aux/IAA destruction occurs via the 26S proteasome (29, 82). Evidence for the involvement of SCF^{TIR1} comes from two complementary experimental approaches (29). First, so-called pull-down assays suggest a physical interaction between SCF^{TIR1} and AXR3 and AXR2. Glutathione S-transferase (GST)–tagged AXR3 or AXR2 proteins were produced in bacteria and added to extracts from plants expressing c-*myc* epitope–tagged TIR1, and glutathione-agarose was used to purify any GST-associated proteins from these extracts. The c-*myc*-tagged TIR1 protein was among the proteins collected in this way, along with other generic SCF components. These data support a direct or indirect physical interaction between Aux/IAAs and SCF^{TIR1}. Second, genetic evidence suggests that this interaction targets the Aux/IAAs for degradation. Support for this idea comes from experiments involving

the amino-terminal half of the AXR3 protein, including domain II and a nuclear localization sequence, fused to the GUS reporter protein and introduced into transgenic plants under the control of a heat-shock inducible promoter. After heat shock, a limited amount of the fusion protein accumulates, but this pool was rapidly depleted in comparison to GUS alone, confirming that fusion to the amino-terminal half of AXR3 can confer reduced protein stability on GUS. However, the stability of the fusion protein was greatly increased when the construct was crossed into a *tir1* mutant background. This suggests that the instability of Aux/IAA proteins requires TIR1 in vivo. Consistent with this idea, the dominant domain II mutations such as *axr3-1* or *axr2-1*, which confer auxin response phenotypes, simultaneously increase the stability of the mutant proteins and reduce or abolish their interaction with SCF^{TIR1}. Hence there is a tight correlation between Aux/IAA protein stability in planta and their ability to interact with SCF^{TIR1} in the pull-down assay (29).

The Effect of Auxin on Aux/IAA Stability

The data described above suggest that auxin signaling requires SCF^{TIR1}-mediated turnover of Aux/IAA proteins. In order to transduce auxin responses, this process must be in some way regulated by auxin. Indeed, exogenous addition of auxin can reduce the half-lives of Aux/IAA proteins below their already low level (29, 112). However, the mechanism by which auxin influences SCF^{TIR1} activity and Aux/IAA turnover is not clear. In theory, auxin could act at a variety of levels by influencing the recognition of the Aux/IAAs by SCF^{TIR1} or the overall activity of SCF^{TIR1} in ubiquitinating the Aux/IAAs. Alternatively, auxin could play a role in transferring the multiubiquitinated Aux/IAAs to the proteasome. Evidence to date is limited but in general supports a role for auxin in regulating the SCF^{TIR1}-Aux/IAA interaction because the auxin-induced destabilization of Aux/IAAs correlates with an increase in the abundance of Aux/IAA-SCF^{TIR1} complexes in the pull-down assay, without affecting the amount of TIR1 present (29). Indeed, the auxin dose response curve for the reduction in Aux/IAA stability and the dose response curve for Aux/IAA-SCF^{TIR1} interaction are remarkably similar (29). These observations suggest that auxin increases the affinity of Aux/IAAs for SCF^{TIR1}. This hypothesis is attractive because it mirrors the established mechanism of action of several similar targeted protein degradation signaling pathways in other species.

In most of the best-understood systems, the interaction of the F-box protein with the degradation target is dependent on the modification of the target, usually by phosphorylation (90). For example, phosphorylation of the NF-Kappa B transcription factors is essential for their recognition by an SCF-type ubiquitin-protein ligase and their subsequent degradation (reviewed in 45). One model then is that auxin regulates a kinase cascade that results in phosphorylation of the Aux/IAA proteins, increasing their affinity for SCF^{TIR1}. There is certainly an increasing body of biochemical and genetic evidence supporting a role for various kinases in auxin signaling. For example, auxin induces a MAP kinase activity in *Arabidopsis* roots (67), Aux/IAA proteins can be phosphorylated in vitro by the photoreceptor phytochrome A (14), and mutations in the PINOID serine-threonine protein

kinase of *Arabidopsis* confer a range of auxin signaling defects (11). However, it is unlikely that direct phosphorylation of Aux/IAAs plays a role in their destabilization because the 13–amino acid region from domain II that is sufficient to confer auxin-inducible destabilization on a reporter protein does not contain any conserved phosphorylation sites (82, 112). Therefore, if the Aux/IAAs conform to the phosphorylation paradigm established in other species, the interaction between the Aux/IAA proteins and TIR1 must be indirect, being mediated by some kind of adaptor protein, with the interaction between the adaptor protein and the Aux/IAA being phosphorylation-dependent.

Although most well-characterized target-SCF interactions are phosphorylation dependent, counter examples are now beginning to emerge. One such example comes from the degradation of the human transcription factor, hypoxia-inducible factor (HIF), by an SCF-related E3 where substrate selection is mediated by the von Hippel–Lindau tumor suppressor protein (VHL) (reviewed in 43). HIF is required for the activation of a variety of responses to hypoxia, which are kept switched off when the oxygen supply is adequate by the rapid destruction of HIF by VHL-mediated ubiquitination. The interaction of HIF with VHL is dependent on the hydroxylation of a HIF proline residue by an oxygen-dependent proline hydroxylase (41, 42). It is tempting to speculate that a similar modification could regulate the interaction of the Aux/IAA degron and SCF^{TIR1} because the most striking feature of the consensus domain II degron is two consecutive, completely conserved proline residues. These prolines are clearly required for Aux/IAA instability because mutations in the prolines are the most common cause of the dominant Aux/IAA stabilizing mutations and are found in alleles such as *axr3-1* and *axr2-1* (72, 83, 84, 93).

Mechanisms of Regulation of SCF^{TIR1} Activity

SCF^{TIR1} ABUNDANCE Apart from regulation of the interaction between the substrate and the SCF complex, the abundance or activity of the SCF complex provides additional possible sites for regulation of the pathway, by either auxin or other interacting signals. The abundance of SCF^{TIR1} can certainly influence auxin sensitivity. Overexpression of *TIR1* in transgenic plants results in increased auxin responses (27). These auxin-response phenotypes are dependent on the additional *TIR1* participating in an SCF complex because overexpression of a *TIR1* variant in which the F-box had been mutated did not result in auxin response phenotypes (27). Furthermore, loss-of-function *tir1* alleles appear to be partially dominant, suggesting haploinsufficiency (85). Therefore auxin responses could in theory be brought about by changes in *TIR1* expression levels. This explanation could account for some of the difference in auxin responsiveness observed between tissues because *TIR1* is not expressed uniformly throughout the plant (27).

RUB1 CONJUGATION TO SCF^{TIR1} Apart from regulation of SCF abundance, SCF activity can apparently be regulated in a variety of ways. New SCF-interacting components have recently been identified and are likely to have regulatory roles.

These components include the SKP1-interacting proteins SGT1 (51) and SnRK protein kinase (23). Another factor that clearly has a dramatic impact on SCF function is the modification of the cullin subunit by the conjugation of a ubiquitin-like protein called RUB1 (homologous to human NEDD8). The conjugation of RUB1 to cullin appears to increase the activity of the SCF (69, 109), and there is increasing evidence that cycles of RUB1 addition and removal are required for efficient SCF function (64, 87). RUB1 is added to cullin by a chain of events very similar to ubiquitination (62). RUB1 is activated by a dimeric RUB1 activating enzyme, the subunits of which are homologous to the amino- and carboxyl-terminal parts of ubiquitin activating enzyme (17, 18, 20, 56, 62). The activated RUB1 is passed to a RUB1 conjugating enzyme and then onto the cullin. It is unclear whether this process requires an E3-like enzyme, however the purified *Arabidopsis* RUB1 activating enzyme and an E2-like enzyme named RCE1 can conjugate RUB1 to AtCul1 in vitro, indicating that an E3-like activity is not essential (18). To date, cullins are the only proteins known to be modified in this way.

Although the precise function of RUB1 modification of cullin is unknown, genetic and biochemical evidence indicates that such modification is essential for wild-type auxin responses. The evidence originates from the analysis of one of the best-characterized auxin response mutants: those in the *AXR1* gene of *Arabidopsis*. These mutants are auxin resistant in every response so far analyzed and show morphological phenotypes consistent with a globally reduced sensitivity to auxin (30, 63, 91, 94). Importantly, Aux/IAA-reporter protein fusions are stabilized in an *axr1* mutant background (29), and *axr1*, *tir1* double mutants have a synergistic phenotype, indicating that AXR1 and TIR1 act in overlapping pathways to destabilize Aux/IAA proteins (85). This hypothesis is supported by the biochemical characterization of *AXR1*. *AXR1* encodes a subunit of a RUB1 activating enzyme, homologous to the amino-terminal half of ubiquitin activating enzyme (60) (Figure 1). In *axr1* loss-of-function alleles, RUB1 conjugation to AtCul1, and hence SCF^{TIR1}, is reduced (17, 20). Some conjugation still occurs, and this is likely to be through the activity of a gene closely homologous to *AXR1* that is found in the *Arabidopsis* genome (17). AXR1 acts in concert with a protein that is related to the carboxyl-terminal half of ubiquitin activating enzyme, named ECR1 (17, 18). The AXR1-ECR1 dimer activates RUB1 by the formation of a thiol ester linkage between RUB1 and a cysteine in ECR1 (20). Transgenic plants overexpressing a mutant form of ECR1, in which this cysteine is replaced by an alanine, show *axr1*-like auxin response phenotypes (17). Presumably the ECR1 cysteine to alanine substitution acts as a dominant negative mutation by titrating out AXR1 into inactive heterodimers. Consistent with this idea, the transgenic lines show reduced conjugation of RUB1 to AtCul1 (17). Taken together, these data indicate that RUB1 modification of SCF^{TIR1} is essential for normal auxin response.

RUB1 REMOVAL FROM SCF^{TIR1} Recent results suggest that removal of RUB1 from SCF^{TIR1} is also important for auxin signaling (87). RUB1 deconjugation is apparently mediated by the COP9 signalosome. The COP9 signalosome is a multiprotein

complex found throughout the plant, fungal, and animal kingdoms, with the notable exception of budding yeast, which has only one of the subunits (reviewed in 86, 104). The COP9 signalosome was originally identified in *Arabidopsis* in screens for light signaling mutants (9). Mutation in any one of the COP9 subunits results in photomorphogenesis even in dark-grown seedlings. In addition to this phenotype, null mutations in the COP9 subunits also result in seedling lethality, but partial loss of function of the CSN5 subunit (also called JAB1) was recently achieved through an antisense approach, and plants carrying the transgene survived to adulthood despite reduced COP9 levels (87). Unexpectedly, the adult phenotype of these plants is reminiscent of the *axr1* mutant phenotype and includes auxin-resistant root elongation, reduced root branching, and increased shoot branching (87). When the antisense CSN5 construct was crossed into the *axr1-3* mutant background, a synergistic effect on the phenotype was observed, suggesting that the COP9 complex and AXR1 act in overlapping pathways (87). Consistent with this idea, the expression of an Aux/IAA-luciferase fusion protein in the CSN5 antisense lines revealed increased stability of the fusion protein compared to its stability in a wild-type background (87). Furthermore, the COP9 signalosome coimmunoprecipitates with SCF^{TIR1}, and AtCul1 interacts with the CSN2 subunit in the yeast two-hybrid system (87). Despite the similarities between the effects of AXR1/ECR1 and COP9 loss of function, paradoxically, COP9 signalosome mutants accumulate AtCul1-RUB1 conjugates, opposite to the effect observed in *axr1* and *ecr1* mutants. This suggests that not only RUB1 addition but also RUB1 removal from AtCul1 is required for full SCF^{TIR1} activity.

POSSIBLE BIOCHEMICAL FUNCTIONS FOR RUB1 CONJUGATION As mentioned above, the precise biochemical function of these proposed cycles of RUB1 conjugation and deconjugation to AtCul1 is not clear. Evidence to date suggests that the primary effect is on SCF activity and not on SCF-target recruitment (64). One possible role could be in subcellular localization. This is suggested by the observation that the cullin found localized to the centrosome of cultured mammalian cells is disproportionately NEDD8 modified (25). Furthermore, conjugation of the ubiquitin-like protein SUMO-1 of humans targets cytosolic RanGAP1 to the nuclear pore complex (70), although other functions for SUMO conjugation have also been identified (reviewed in 66). SCF activity and ubiquitination of Aux/IAAs requires nuclear localization, but it is possible that the degradtion occurs in the cytoplasm or in a nuclear subcompartment. Therefore, RUB1 modification could be required for subcellular targeting of the SCF. However, so far the data do not support this hypothesis because AtCul1 appears to be normally localized in the nucleus in *axr1* mutant plants where RUB1 conjugation is severely compromised (17).

An alternative hypothesis is that RUB1 conjugation is involved in mediating SCF-proteasome interactions. In this context it is interesting to note that the subunits of the COP9 signalosome, with which the SCF interacts, are homologous to the subunits of the lid subcomplex of the 26S proteasome (105). Evidence that the COP9 signalosome interacts with the proteasome also exists (55).

The activity of the RUB1 conjugation pathway in *Arabidopsis* is apparently unaffected by auxin addition, indicating that it is not a direct route for auxin signal transduction (17). Indeed, the fact that most, if not all, of the *axr1* phenotypes can be explained in terms of reduced auxin response is something of a mystery because it appears that AXR1 is involved in conjugation of RUB1 to all SCF complexes. Why then do *axr1* mutants show such specific auxin-response phenotypes? It seems likely that this reflects the particular sensitivity of auxin-regulated protein degradation to RUB1 modification of cullins. There is certainly evidence from yeast that different pathways are varyingly sensitive to loss of RUB1 modification activities. In fission yeast, the RUB1 conjugation-deconjugation pathway is essential for viability (71, 76). In contrast, budding yeast lacks most of the COP9 signalosome subunits (71), and the RUB1 conjugation pathway is not required for viability (56, 62). However, RUB1 conjugation is necessary for efficient functioning of SCF^{CDC4}, with other SCFs being apparently unaffected (56).

Aux/IAA Protein Function

Whatever the mechanisms for auxin input into the system, the auxin-regulated degradation of Aux/IAA proteins is an essential part of the auxin response. An important question then is what are the effects of the auxin-induced changes in Aux/IAA protein abundance?

DIMERIZATION WITHIN THE AUX/IAA FAMILY Aux/IAA proteins appear to act as transcriptional regulators through the formation of a variety of dimers. First, they can dimerize with other members of the Aux/IAA family (46). These interactions require domains III and IV, and possibly domain I (Figure 2) (46, 77). In *Arabidopsis*, there are at least 24 different Aux/IAAs (83). Although only a subset of Aux/IAAs are expressed in any one tissue (1) and not all Aux/IAA family members may dimerize with high affinity, the possibility of an enormous number of different Aux/IAA dimers still exists. The function of Aux/IAA dimers is not clear. Domain III and the surrounding region have homology to bacterial transcriptional repressor proteins of the beta alpha alpha class (68). Dimerization of such proteins results in the formation of an unusual sequence-specific DNA binding domain based on beta-sheet. It is therefore possible that Aux/IAA proteins can bind DNA directly and regulate transcription. Their nuclear location is certainly consistent with this idea, although to date there is no published evidence to support it directly.

DIMERIZATION BETWEEN AUX/IAAs AND AUXIN RESPONSE FACTORS (ARFs) A second possibility is that dimerization of Aux/IAAs within the Aux/IAA family prevents dimerization of Aux/IAAs with other partners. The only other protein family known to be able to heterodimerize with Aux/IAAs is the auxin response factor (ARF) family of transcription factors (46, 97, 99) (Figure 2). ARFs interact with Aux/IAAs through carboxyl-terminal domains with homology to Aux/IAA domains III and IV. ARF proteins can certainly bind DNA directly through an

amino-terminal B3-type DNA binding domain, similar to that found in the maize VP1 protein (97, 99). The ARFs tested to date bind specifically to the TGTCTC-containing auxin response element (ARE) of auxin-regulated genes. Synthetic palindromic or direct repeats of these six nucleotides are sufficient to bind ARFs and confer auxin regulation on the transcription of a reporter gene (97–99). Hence ARFs appear to mediate auxin-regulated gene expression through binding to AREs and Aux/IAAs have the potential to alter the transcription of auxin-regulated genes through interacting with ARFs.

ARFs also form a large gene family, consisting of at least 10 members in *Arabidopsis* (99). Much of the work to characterize the effects of ARFs and Aux/IAAs on transcription from auxin-regulated promoters has been carried out using a carrot cell suspension culture protoplast system in which ARF or Aux/IAA-derived genes are coexpressed with a synthetic or natural auxin-inducible promoter-reporter fusion (98, 100). Expression of ARFs in this system has produced an interesting range of results (98). ARFs can be grouped into subfamilies depending on their effect on gene expression in this system, which correlates with their predicted protein sequence between the amino-terminal DNA binding domain and the carboxyl-terminal dimerization domains. Expression of ARF1, which is P/S/T-rich in this region, suppresses both basal and auxin-inducible expression from ARE-containing promoters. In contrast, expression of ARF5, ARF6, ARF7, or ARF8, which are Q-rich in their middle regions, increases both basal and auxin-inducible expression from the ARE-containing promoters, whereas expression of ARF2, ARF3, ARF4, or ARF9 has no effect. These regulatory characteristics are independent of the ARE binding because essentially identical results were obtained when the ARF DNA binding domain was replaced with the DNA binding domain from the budding yeast GAL4 protein and the ARE-reporter gene fusion was replaced simultaneously with a GAL4 binding element-reporter gene fusion (98). These data suggest that auxin inducibility of ARE-containing promoters depends on the middle and C-terminal regions of the ARFs because a Q-rich middle region with a C-terminal Aux/IAA-like dimerization domain is sufficient to confer auxin inducibility on a GAL4 DNA binding domain-promoter element system.

When the DNA binding domain was removed from the various ARFs, and these truncated versions were introduced into a carrot protoplast with an ARE-reporter construct, the same effects on transcription as with the full-length proteins were observed (98). These effects were abolished if the dimerization domains were also removed (98). One explanation for these results is that the truncated ARFs can dimerize with endogenous ARFs that occupy the AREs through their DNA binding domains, and their middle regions can subsequently regulate transcription. Certainly, domain-swapping experiments suggest that the middle region determines whether an ARF will activate or repress transcription, and all these effects are dependent on the dimerization domains because expression of the middle regions alone did not affect transcription from the ARE-regulated reporter (98).

In a similar result using this assay, expression of several Aux/IAA family members has been shown to inhibit auxin-inducible transcription from ARE-containing

promoters (100). This result is consistent with the idea that Aux/IAA dimerization with ARE-bound ARFs can prevent the ability of ARFs to active transcription in response to auxin. An attractive hypothesis is that this occurs by Aux/IAAs competing with ARFs for dimerization through domains III and IV. However, the presence of endogenous ARFs on the AREs in this system has not been proven, and there is currently no direct evidence to show ARF-Aux/IAA association on a promoter.

A MODEL FOR AUXIN-REGULATED GENE EXPRESSION Despite the caveats, these results have led to a model to explain auxin-regulated transcription from ARE-containing promoters (Figure 3). In this model, it is proposed that ARFs permanently occupy the AREs of auxin-regulated genes, regardless of auxin levels. When auxin levels are low, Aux/IAA proteins are stable, and they dimerize with ARF proteins on the AREs, blocking ARF function. When auxin levels rise, the

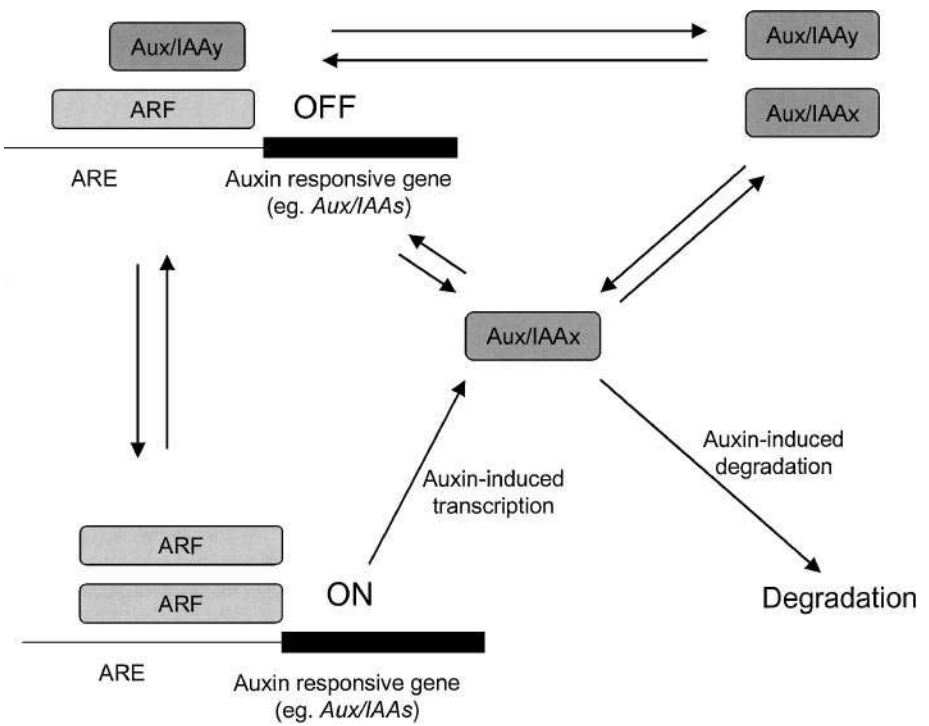

Figure 3 A model for Aux/IAA protein action. Aux/IAA proteins are able to form a variety of dimers both within the Aux/IAA family and with members of the ARF family (*top*). The equilibrium between these dimers has the potential to regulate transcription from ARE-containing promoters. The abundance of any one member of the Aux/IAA family (e.g., Aux/IAAx; *center*) is regulated by transcription and degradation, both of which are regulated by auxin (*bottom*). The dynamics of this regulatory network are clearly very complex.

Aux/IAAs are destabilized, and the resulting drop in Aux/IAA abundance allows ARF-ARF dimerization at the AREs and hence auxin-regulated transcription of the associated genes.

Although there is little direct evidence to support this model, a large body of compelling circumstantial evidence is accumulating. In addition to the work described above, mutations in two of the Q-rich ARFs have been recovered, and these confer a range of auxin-related phenotypes including reduced expression from ARE-containing auxin-inducible promoters (32, 33). This is consistent with the idea that Q-rich ARFs are required to activate expression of auxin-inducible genes in vivo. There is also good evidence that transcription from ARE-regulated genes is usually kept inactive by very unstable auxin-inactivated repressor proteins because transcription from ARE-containing promoters can be activated by either auxin or inhibitors of protein synthesis such as cycloheximide (53). The possibility that these unstable transcriptional inhibitors are the Aux/IAA proteins is supported by the observation that the dominant, stabilizing mutations in domain II of the Aux/IAA proteins often result in the constitutive repression of transcription from auxin-inducible ARE-containing promoters (1, 72, 75, 83).

Although this model is compelling, it is very much complicated by the fact that most *Aux/IAA* genes contain ARE elements in their promoters and are consequently auxin inducible at the level of transcription (reviewed in 3, 31). In fact, the *Aux/IAA* gene family was originally defined because of this auxin inducibility, with some members being induced within a few minutes of auxin addition (reviewed in 3, 31). Hence at the same time as their protein levels are being depleted by auxin-induced SCF^{TIR1}-mediated destabilization, they are being replenished by increased transcript accumulation. The auxin-induced increases in transcript levels often persist for many hours and outlast more modest increases in Aux/IAA protein levels (1, 74). This is not the pattern of message and protein accumulation that would be expected if Aux/IAA proteins repress their own transcription. On the contrary if this were the case, then Aux/IAA mRNA levels should decrease as Aux/IAA protein levels rise.

However, these predictions do not take into account the fact that all Aux/IAAs are different, with each Aux/IAA protein potentially regulating the transcription of a specific subset of *Aux/IAA* and other auxin-responsive genes. For example, the transcription of a very rapidly induced *Aux/IAA* gene may be inactivated by the product of an *Aux/IAA* family member that is induced much later after the increase in auxin levels. Such specificity of Aux/IAA gene function is demonstrated by several lines of evidence. First, very similar stabilizing mutations in different Aux/IAA genes confer quite different and even opposite phenotypes. For example, the *axr3-1* mutant has increased adventitious rooting, whereas the *axr2-1* mutant has fewer adventitious roots than wild type (72). Furthermore, the transcriptional inductions of different Aux/IAA family members show different temporal patterns, different auxin-dose response kinetics, and different tissue specificities (1). The transcription of some members requires new protein synthesis, and some are not even auxin induced (1, 83). In addition, if there is competition for dimer formation both within

and between the Aux/IAAs and the ARFs, then depending on the relative affinities of particular protein combinations, increased levels of some Aux/IAAs could increase Aux/IAA-Aux/IAA dimerization at the expense of Aux/IAA-ARF dimerization. Hence ARF-ARF dimerization might be increased (Figure 3). In support of this idea, stabilizing mutations in some Aux/IAAs appear to result in increased auxin responses, including ectopic activation of auxin-inducible genes (13, 52, 61).

In *Arabidopsis*, there are at least 24 Aux/IAAs, which can have different expression patterns, half-lives, auxin-induced destabilization dynamics, and dimerization affinities, and at least 10 ARFs with similar variations in patterns of expression, effects on transcription, and dimerization affinities. Hence when auxin levels change, the resulting changes in Aux/IAA stability can trigger a mind-boggling array of possible effects on transcription. In this way, this model for auxin response perhaps can go some way toward explaining how such diverse responses can be induced by a simple signal, acting through a single signaling pathway. The diversity of possible responses is expanded still further by considering the possibility of additional degradation targets for SCF^{TIR1} and its homologues. For example, the stability of the auxin efflux carrier PIN2/EIR1 appears to be regulated by auxin in an AXR1-dependent manner (89).

CONCLUSIONS

These recent advances provide a framework in which to understand auxin signaling. They highlight four key challenges remaining. First, a better characterization of the events that link auxin to changes in Aux/IAA stability is required. Second, although the current model encompasses sufficient complexity to allow for diverse responses to auxin, the exact manner in which this complexity is encoded to produce each specific auxin response is still unknown. Cracking this code will require an understanding of which Aux/IAAs and ARFs are involved in each auxin response and of the changes in interactions that occur between them in response to changing auxin levels and hence changing Aux/IAA levels. Third, it will be necessary to determine the mechanisms by which the resulting changes in gene expression mediate specific auxin responses. Fourth, some auxin responses are likely to be independent of the SCF system, and these alternative pathways also need to be investigated.

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